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The hMOF protein is a chromatin-modifying factor. Chromatin structure plays a critical role in gene expression. Since hMOF has a chromodomain region as well as acetyl trasferase activity, its inactivation can influence modification of chromatin during DNA metabolism. The proposed experiments of this grant proposal will determine functions of hMOF gene. This will be achieved by generating isogenic cells with and without hMOF function. Both in vivo and in vitro experiments will be performed to determine the function of hMOF in context with radioresponsiveness and oncogene transformation. If hMOF proves to be involved in the radioresponsiveness and neoplastic transformation, then the clinical implications of this proposal are highly significant. It may, in the future, be prudent to screen each breast cancer patient prior to any final therapeutic decision. This will be accomplished through the use of quantitative RT-PCR and the test results can be obtained within a day. There are several benefits of identifying an individual's normal tissue with loss of hMOF gene expression. First, it will allow us to prospectively identify the sensitive subset of patients. Second, the radiosensitive patients will be taken for an alternative therapy if exist and would be spared a great deal of suffering. Third, it will be possible that once we identify a subset of patients that show a genetic basis of radiation sensitivity, the radiation dose to the remaining breast patients could be increased to be more effective for local tumor control. Fourth, it will provide health professionals a molecular diagnostic approach to predict the suitability of an individual for radiotherapy.

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# Introduction:

Most patients with breast cancer tolerate radiotherapy well with only limited acute, reversible adverse effects. However, about 5% of patients experience severe, delayed complications such as skin pigmentation changes, subcutaneous fibrosis, rib fractures, cardiac disease, pulmonary fibrosis, second primary cancer (specifically esophageal squamous-cell carcinoma as well as adenocarcinoma) and other complications, which manifest several years after treatment with ionizing radiation. Epidemiological studies have shown that irradiation of the breast especially among young women, increases the risk for subsequently developing breast cancer. It might thus be expected that genes that are known to influence radiation sensitivity may be associated with the radiotherapy related adverse effects. The human genes that have been found to be responsible for ionizing radiation sensitivity are *ATM* (ataxia telangiectasia mutated), *BRCA1*, *BRCA2, NBS1,* etc. Mutations in *BRCA1* and *BRCA2* contribute to about 15% of familial breast cancer risk and their contribution to sporadic breast cancer is very low. In such cases, genes frequently altered in the general population, e.g., *ATM* may be an important risk factor. However, screening for *ATM* mutations in sporadic breast cancer cases has not revealed the magnitude of involvement of the *ATM* gene expected. Since ATM as well as BRCA1 have been reported to interact with chromatin modifying factors, it is possible that such factors may be involved in the radiation-induced morbidity. Therefore, there is a need for the identification of chromatin modifying factors involved in ionizing radiation sensitivity, genomic instability and carcinogenesis.

## Body

### Specific Aims:

The goal of this proposal is to understand the mechanisms underlying radiosensitivity. Two specific questions are being addressed in this grant application: (1) Whether hMOF is involved in ionizing radiation (IR) response and; (2) Whether hMOF is involved in pathobiology of the breast cancer. We proposed to complete the following aims: (1) To determine whether mutations in the *hMOF* gene correlate with ionizing radiation sensitivity. (2) To generate MOF knockout mice in order to determine the pathobiology of gene. (3) To determine whether ionizing radiation enhances neoplastic transformations in mouse embryonic fibroblasts of MOF knockout mice. MOF knockout mice will also be examined for spontaneous as well as IR-induced tumor formation.

### Studies and Results during first year of funding:

During the first year, we have addressed the specific aim 1. This specific aim allowed us to determine whether inactivation of hMOF leads to enhanced cell killing by IR treatment. We cloned the full length cDNA as well as fragments of hMOF and expressed in human cell line.

***Task 1*** To determine whether mutations in the *hMOF* gene lead to IR sensitivity:

a. *"Develop a series of plasmids for expressing full length and different domains of hMOF open reading frame. Point mutations within specific domains will be made. All changes in the gene will be confirmed using DNA sequencing. (Months 1-9):".* We have made deletion constructs and completed more than 50% of this task (Fig. 1).

*Fig. 1: Strategy for cloning the hMOF and its domains. hMOF protein is denoted at the top with a line representing 458 amino acids (a.a). The second line represents a diagram of hMOF cDNA encoding full length hMOF protein with three domains:* ***cd*** *stands for the chromodomain region,* ***zf*** *stands for the zinc finger, and* ***ac*** *for the acetyl Co-A binding site. Five different fragments were generated by PCR using specific primers. All clones are drawn to approximate scale. Each fragment is cloned in the pBABE retroviral vector. At the bottom is the pBABE retroviral vector is subcloned with either of the five cDNA fragments. Oligonucleotides that are used for RT-PCR analysis are shown as primers 1 to 4. Either primer 1 or 3 are used for RT in the absence or presence of Superscript RT. Primer pairs 2 and 3, 2 and 1, 4 and 1, or 4 and 3 are be used for cDNA amplification, as indicated.*

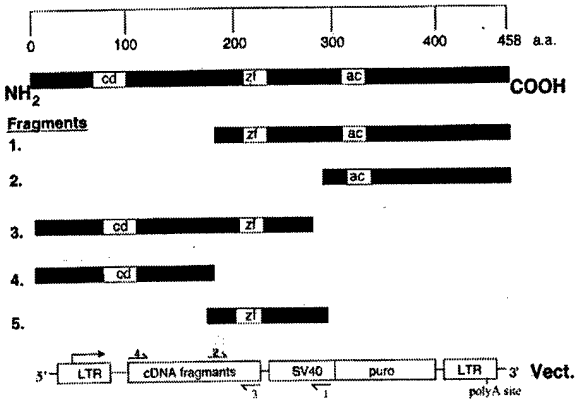


Fig. 1

b. "*Antibodies will be raised against hMOF protein. These will be tested for immunostaining, immunoprecipitation and immunoblotting (Months 1-6)*". We have raised several antibodies against hMOF and one of them works pretty well for westerns. We determined the hMOF levels in different cell lines by using anti-hMOF antibody (Fig. 2).

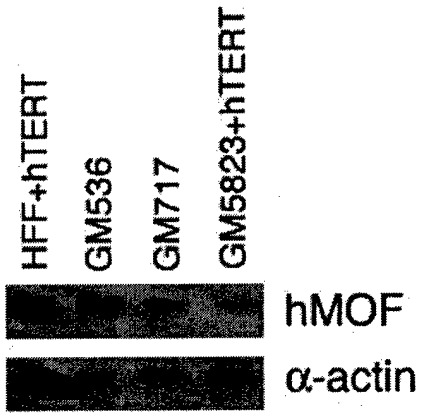


Fig. 2

*Fig. 2: Western analysis of hMOF in A-T and normal cells with anti-hMOF antibody. Note hMOF levels are lower in A-T cells (GM5823+hTERT, GM717) when compared to control cells (HFF+hTERT, GM536).*

c. "*Cells will be transfected with vectors containing either complete opening reading frame of hMOF or mutated hMOF to develop stable cell lines (Months 3-9)*". We have developed one cell line expressing a fragment of hMOF containing chromodomain region.

c. "*Assays will be performed to ascertain which fragment of hMOF influences the IR response. Cell survival will be performed by clonogenic assay. DNA double strand break repair will be performed by neutral elution as well as field gel electrophoresis assay. Chromosomal aberration after treatment with ionizing radiations will be determined by premature chromosome condensation technique and metaphase scoring (Months 6-18)*". We have done survival and chromosome damage repair assays after treatment with IR. We found that the expression of mutant hMOF enhanced cell killing after IR treatment (Fig. 3). Furthermore, we found that cells expressing hMOF fragment had higher residual chromosome damage after IR treatment (Fig. 4).

**Fig. 3.** *Survival after IR treatment. Dose-response curves are shown for normal cells with empty vector (HFF+hTERT), a normal fibroblast with ectopic expression of mutant hMOF gene (HFF+mutant hMOF) and A-T fibroblasts (GM5823+hTERT) treated with IR. A-T human fibroblasts were used as a positive control to indicate radiosensitivity. Note: Normal cells with mutant hMOF expression display more cell killing by IR treatment when compared to their parental normal cells.*

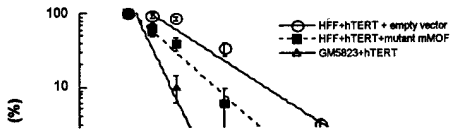


Figure 3

**Fig.** 4. *G2 type chromosomal aberrations after IR treatment. Cells in exponential phase were irradiated with 1 Gy. Metaphases were harvested after 75 min following irradiation and examined for chromosomal aberrations. The frequency of chromosomal aberrations was higher in cells expressing hMOF fragment (HFF+hTERT+mutant hMOF).*

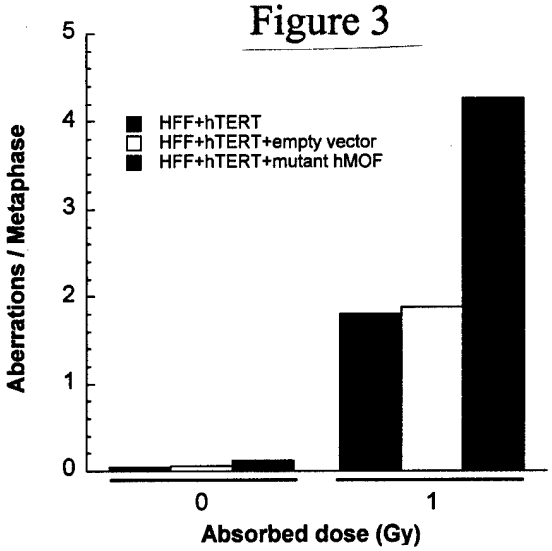


Figure 4

d. "*Determine the role of hMOF on cell cycle checkpoints after treatment with IR. Cell cycle analysis, levels of p53 and status of its ser-15 phosphorylation will be determined. Cell cycle analysis will be done by Flow cytometry as well as premature chromosome condensation procedure. Levels of p53 and status of its ser-15 phosphorylation will be done by the immunoprecipitation of p53 protein and immunoblotting with antibody against ser-15 phosphorylation (Months 15-20)*". We have started to analyze the influence of hMOF inactivation on p53 status after IR treatment.

- **Key Research Accomplishments**
- We developed one good reagent, which has a great potential for their utility in the research fields of radiation biology in specific and cancer biology in general. This reagent is the development of the antibody against the hMOF.

- We showed for the first time that hTERT interacts with telomeres and some novel function of hTERT in addition to its role in telomere maintenance (Sharma et al., 2003a).

- We have demonstrated that isoforms of human heterochromatin protein 1 (HP1) influence telomere stability, DNA repair and cell killing after IR treatment. The results have appeared in Molecular and Cell Biology (Sharma et al., 2003b).

- We showed that expression of mutant hMOF enhances IR-induced cell killing as well as higher chromosome damage (Fig. 3, 4).

- We cloned mouse MOF gene and its targeting vector to generate the Mof knockout mice.

**c. Reportable Outcomes**

1. We developed antibody for hMOF.
2. Cloned mouse MOF gene and prepared targeting vector.
3. Characterized the functions of MOF gene.

**Conclusions: Plans for next year (2003-2004):**

During the second year, we will complete the work proposed under task 2.

***Task 2.*** To generate MOF knockout mice in order to determine the pathobiology of gene.

To determine the pathobiology of *hMOF* gene, we will generate *Mof* knockout mice. We have cloned and sequenced a full-length mouse *Mof* cDNA. To isolate an isogenic *Mof* mouse gene for construction of the targeting vector, we screened a genomic λ phage library from the mouse strain 129/Sv (Stratagene) using Mof cDNA as a probe and obtained three overlapping positive clones containing exons 1-4 of the *Mof* gene. We identified 4 and 9 kb EcoRI fragments of *Mof*.

We will construct a homologous recombination targeting vector that will replace exons 1-3 of the *mMof* gene encoding the MET initiation site and the chromodomain region. The targeting vector, pmMof, will be linearized at the unique Not 1 site then electroporated into WW6 embryonic stem cells. Genomic DNA from the resulting colonies will be screened for targeted recombination by PCR analysis using the primers. Positive clones will be confirmed by diagnostic Southern blot analysis of HindIII restricted genomic DNAs. Positive cell lines will be injected into C57BL/6J blastocysts and chimeric mice with significant ES cell contributions identified by coat color by the Embryonic Stem Cell Facility of the Siteman Cancer Center, Washington University School of Medicine. Germ line transmission of the deleted mMof allele will be detected in F1 agouti offspring of male chimeras and C57BL/6J females by the presence of

the 7.5 kb HindIII fragment in Southern blot analysis of tail DNA. Mating heterozygous F1 animals will produce homozygous mMof knock out mice. We have experience in generating homozygous null mice from heterozygous animals. Analysis of F2 offspring should result in approximately 25% wild type, 50% heterozygous and 25% homozygous mutants. If such a ratio of animals is observed, it will indicate that the gene does not cause embryonic lethality.

If global ablation of Mof function in the mouse may result in early embryonic lethality, we will construct a targeting vector for conditional mutagenesis, which will allow the global and the tissue-specific inactivation of *Mof* (if necessary). Currently the *cre/loxP* strategy is probably the most applied system of conditional mutagenesis. Recent advances with the conceptually related *Flpe/FRT* system offers an alternative, and the two systems can be combined advantageously. The *cre/loxP* system requires the generation of two strains of mice. In one of them, the *Mof* sequence to be deleted upon recombination will be flanked by *loxP* sites ($Mof^{flox}$) introduced by homologous recombination in embryonic stem cells. The second mouse strain carries the *loxP* site-specific *cre* recombinase under control of a temporal- or tissue-specific promoter of choice.

**Publications:**

We have achieved about 80% of envisaged goals for the first year of this grant. During the current funding period 7 papers were published, 3 are in press and 1 is submitted for publication. Each paper contributed to the over all goals of the proposal.


1. Sharma G.G, Gupta A., Scherthan H., Dhar S., Wang H., Gandhi V., Iliakis G., Young C.S.H., and **Pandita T.K.** hTERT Associating with Telomeres Reduces Spontaneous Chromosome Damage and Enhances DNA Repair. Oncogene 22:131-146 (2003a).
2. **Pandita T.K.** Telomerase and Radiosensitivity of Human Tumors. Telomeres and Telomerase: Cancer and Biology. Ed. Guido Krupp and Reza Parwaresch. Pp. 30-46 (2003).
3. **Pandita T.K.** and Roti Roti JL. Role of Telomerase in Radiotherapy. Oncology Reports 10:263-270 (2003).
4. **Pandita T.K.** A multifaceted role for ATM in genome maintenance. Exp. Rev. Mol. Med. 5:1-21 (2003).
5. Sarkar D, Leszczyniecka M, Kang D, Lebedeva IV, Valerie K, **Pandita T.K.** and Fisher PB. Down-regulation of Myc as a potential target for growth arrest induced by human polynucleotide phosphorylase ($hPNPase^{old-35}$) in human melanoma cells. JBC 278:24542-24551 (2003).
6. Sharma G.S., Hwang K.K., Pandita R.K., Gupta A., Worman H.J., Wellinger R.J. and **Pandita T.K.** Human Heterochromatin Protein 1 Isoforms $HP1^{Hs}$- and $HP1^{Hs}$- Interfere with hTERT-telomere interactions and correlates with changes in cell growth and ionizing radiation response. MCB 23: 8363-8376 (2003).
7. Sharma G.G., Hall E.J., Dhar S., Gupta A, Rao P.H. and **Pandita T.K.** Telomere stability correlates with longevity of human beings exposed to ionizing radiations. Oncology Reports 10: 1733-1736 (2003).
8. **Pandita T.K.** Detecting influence of cell cycle regulatory proteins on human telomeres. Methods in Molecular Biology 241: (in press) (2003).
9. **Pandita T.K.** Enrichment of cells in different phases of cell cycle by centrifugal elutriation. Methods in Molecular Biology 241:(in press) (2003).
10. Hunt C.R., Dix D.J., Sharma G.G., Pandita R.K., Gupta A., Funk M. and **Pandita T.K.** Genomic instability and enhanced radiosensitivity in Hsp70.1/3-deficient mice. MCB (in press) (2003).


**Appendix: None**